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Inclusion of Biomarkers for Detecting Perturbations in the Heart and Lung and Lipid/Carbohydrate Metabolism in National Toxicology Program Studies

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Abstract

Environmental factors and exposures may contribute to many serious diseases afflicting humans. Biomarkers are useful to understand disease processes and identify early events leading to disease. The National Toxicology Program (NTP) convened a workshop in September 2006 to help identify biomarkers that could be used in toxicology studies with rodents to predict disease outcome and detect early events in disease processes. Expert scientists reviewed biomarkers for disease/injury related to the heart, lung, and/or changes in lipid/carbohydrate metabolism, and made recommendations for those that could be incorporated into NTP studies on a routine or selective basis. Although numerous biomarkers were discussed, only a few were considered amenable for routine use. This article summarizes recommendations for the most promising biomarkers and presents the NTP perspective on those that will be included in the bioassay program on a routine or special study basis. Breakout group reports and additional information on the workshop, including participants, presentations, and background materials, are posted on the NTP website *http://ntp.niehs.nih.gov/go/20940*.

Introduction

Inherited susceptibilities, environmental factors, and age play a role in the development of major diseases (Schwartz *et al.*, 2004) and the National Toxicology Program (NTP) has played an important role in identifying the environmental factors that contribute to these diseases. While probably best known for its cancer bioassay program, the NTP also conducts studies to address other diseases and disorders (i.e., reproduction toxicity, immunotoxicity, neurotoxicity, etc.) and is interested in enhancing its assessment of environmental influences on other major diseases. Heart disease, respiratory disease, and disorders of metabolism such as diabetes consistently rank in the top 10 leading causes of morbidity and mortality for both men and women in the United States and have significant associated personal and financial costs. For these reasons, the NTP is in the process of identifying and incorporating biomarkers for diseases of the heart, lung, and lipid/carbohydrate metabolism to enhance its toxicology testing program. It is important to emphasize that while NTP studies have a default or "core" selection of endpoints that are consistent across studies (see "NTP Studies" in background materials at *http://ntp.niehs.nih.gov/go/28624* and Table 1), the majority of studies also include adjunct components ranging from the collection of additional selective endpoints to the use

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of novel technologies. For example, in a study of ephedra (a dietary supplement) telemetry was utilized

Table 1. Routine Endpoints for the Evaluation of Heart, Lung and Lipid/Carbohydrate Metabolism

	Current	Proposed Additions
Heart	histopathology	Troponin
Lung	histopathology	Selective use of BAL endpoints
Lipid/Carbohydrate	serum glucose, histopathology (e.g., liver, pancreas, adrenal, gastrointestinal tract, and thyroid)	Serum cholesterol, triglycerides and fructosamine

to measure cardiovascular responses (Howden *et al.*, 2005). The NTP is also assessing and evaluating enhanced *in vivo* test protocols for assessing QT interval prolongation. While these measurements are not typical, a major goal of the workshop was to identify endpoints that could be added *routinely* to toxicity studies to provide more confidence that NTP studies are adequately screening for changes in heart and lung disease/function and lipid and carbohydrate metabolism.

On September 20-21, 2006, the NTP organized a workshop "Biomarkers for Toxicology Studies" to help address this task.[1] The specific purpose of the workshop was to identify biomarkers related to heart, lung, and lipid/carbohydrate metabolic function and injury that could be included in subchronic (90-day) rodent toxicology studies to better characterize endpoints of environmentally-induced disease or biological processes related to human disease etiology. This workshop is the fourth in a series the NTP has organized as part of implementing the NTP Roadmap to critically evaluate its testing program and determine whether any refinements or new strategies are needed to maximize its impact on public health (*http://ntp.niehs.nih.gov/go/vision*).

An ideal biomarker should qualitatively or quantitatively measure biologic, pathologic, or pharmacologic responses (De Gruttola *et al.*, 2001), and be a specific and sensitive indicator of a disease process (Kraemer, 1992). Biomarkers may measure upstream events prior to the onset of a disease or downstream disease events. Biomarkers may measure nonspecific biologial variations or adverse effects characteristic of disease processes. For NTP purposes, biomarkers can be used to (1) improve detection of disease and disease processes, (2) maximize the information derived from toxicology studies used for hazard identification, (3) aid in understanding mechanisms of disease processes, and (4) detect changes early in disease development. This includes not only biomarkers for specific diseases, but also biomarkers that measure common mechanisms in multiple disease processes. The ideal biomarker would also need to be both appropriate to measure disease in model systems (e.g., rodents) and predictive of an analogous disease process or altered function in humans. The biomarker may be the same across species (e.g., insulin, troponin) or have an analogue in rodents and humans (e.g., $\alpha 2$-macroglobulin in the rat and C-reactive protein in humans). Additional desirable biomarker attributes include the ability for samples to be easily collected and assays that can be conducted in a timely and cost-effective manner.

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[1] The workshop agenda, presentations, background materials, roster of the invited panel and other attendees, and other related information can be found on the NTP website (*http://ntp.niehs.nih.gov* see "Meetings & Workshops" or directly at *http://ntp.niehs.nih.gov/go/28624*).

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Prior to the workshop, NTP staff summarized candidate disease biomarkers from a review of the literature (see topic specific worksheets in "Background Materials" at *http://ntp.niehs.nih.gov/go/20940*) that included a broad look at the field of biomarkers in physiological measurements, serum and tissue analyses, and noninvasive techniques (e.g., imaging). An expert panel from academia, industry, and government was convened for each topic (heart, lung, lipid/carbohydrate). Following a plenary session that provided an overview of the biomarkers for each topic, workshop participants met in their respective discussion groups to identify the most useful biomarkers that NTP could consider incorporating into its studies. Discussions focused not only on biomarkers for specific diseases, but also biomarkers that measure common mechanisms in multiple disease processes such as inflammation. The following summaries focus on breakout group discussions. For complete details of the proceedings, see the meeting website (*http://ntp.niehs.nih.gov/go/20940).*

Biomarkers of Lung Function and Injury

The lung breakout group discussed a variety of biomarkers and approaches for collecting biomarkers ranging from bronchoalveolar lavage (BAL) fluid analysis, respiratory function, enhanced tissue pathology, imaging, and gene analysis to proteomics. Of these, the group considered BAL and enhanced histopathology as potentially the most useful for the NTP. Gene expression analysis and use of imaging techniques were also considered promising for detecting the broad range of lung disease. However, none of the approaches were considered appropriate for routine use for a variety of reasons including the possibility that they would necessitate the use of additional animals, issues regarding data interpretation, and/or requirements for specialized equipment.

BAL is applicable to both rodents and humans, can identify early as well as late events in lung disease/dysfunction, and does not require specialized or new technology for analysis. BAL analysis can be performed readily at necropsy and coupled with standard histopathological analysis for detecting pulmonary injury. The group felt BAL would be especially appropriate for identifying changes in the lungs by obtaining cell counts and differentials but did not make specific recommendations on which other endpoints should be assessed. BAL endpoints that help to identify lung inflammation and injury include total protein, beta-glucuronidase, lactate dehydrogenase, alkaline phosphatase, chemokines, cytokines, antioxidants, and albumin) (Benson *et al*, 1989; Henderson, 2005; March *et al*, 2006; Seagrave *et al.*, 2005). Because the method is largely invasive in small animals and would be most informative when conducted at multiple time points, a major drawback is that use of lavage analysis would require additional animals be added to a study.

Enhanced histopathological analysis can identify lung injury, inflammation, apoptosis, repair, and other events either early or late in the disease process. Special staining techniques identify specific marker proteins allowing for quantitative analysis and potentially detection of exposure-related toxicity. For example, trichrome and Periodic

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Acid Schiff (PAS) stains are used to identify collagen and mucopolysaccharides, respectively, and proliferating cell nuclear antigen (PCNA) and Ki67 protein may be used to measure cell proliferation (Meert *et al.*, 2004). Electron microscopic analysis is useful for identifying mitochondrial damage (Bishop *et al.*, 2004; Dunnick *et al*, 2004) . Morphometric analysis of the lung allows for quantitative assessment of lesions, but requires accurate measurement of lesion size and is labor-intensive (March *et al.*, 2005). In addition, immunohistochemical analysis of secreted proteins or protein products associated with tissue injury assessed in BAL can be useful for cross-platform confirmation purposes.

Imaging using x-ray, magnetic resonance imaging (MRI), or positron emission tomography (PET) scanning for lung injury holds promise for detecting early stages of lung disease, has the advantage of being non-invasive (Chen *et al*, 2000) , and would allow for comparison of animal/human disease (Bakker *et al.*, 2005). The major advantages of imaging are that it is non-invasive, allows for the evaluation of multiple organs simultaneously, and can be conducted repeatedly in the same animal. Major problems, however, are that imaging techniques have limited capacity to detect subtle or scattered focal tissue change and the relatively high equipment costs preclude the use of imaging technologies on a routine basis.

Biomarkers of Heart Function and Injury

The heart breakout group recommended the routine inclusion of three biomarkers into NTP subchronic studies: troponin (McDonough and Van Eyk, 2004; Wallace *et al*, 2004), α2-macroglobulin in the rat, and B-type natriuretic peptide (BNP) (Apple *et al.*, 2005a; Apple *et al*, 2005b) . All of these biomarkers are considered *indicative* rather than *predictive* of a disease process. Imaging techniques for suspected cardiotoxicants were also considered promising as the technology becomes available but were not currently recommended.

Troponin T and I are components of the heart muscle and their release into serum is indicative of early events in heart tissue degeneration, necrosis, and myocyte damage (Wallace *et al*, 2004) . In humans, troponin (T or I) isthe preferred marker for diagnosis of myocardial injury and increased cardiac troponin is defined as a measurement greater than the 99th percentile of an appropriate reference group. In animal models, a cardiac troponin response is heart-specific and often associated with morphological changes histologically. Troponin can be measured from a serum sample, thus obtaining it is a relatively non-invasive procedure. There are several drawbacks associated with the use of troponin as a biomarker. The selection of an appropriate assay method is very important. Many reagent kits are available from various manufacturers to measure troponin I but they do not perform equally in laboratory animals (Jaffe *et al.*, 2006). Time dependence issues also exist since the circulating half-life is short (several hours) in rodents. Lastly, the assay has a relatively high cost per animal.

The group recommended acute phase reactant protein, α2-macroglobulin (analogous to human C-reactive protein) is not cardiac-specific It was recommended because they felt it important to have an indicator of systemic inflammation (Zhang *et al.*, 2006). α2-

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Macroglobulin is considered to be a "negative" predictor such that a normal value indicates the absence of systemic inflammation. In a non-specific way, α2-macroglobulin may also address potential effects on the vasculature. While no good markers of vascular damage were identified, an elevated α2-macroglobulin could be associated with vascular injury including inflammation. Conversely, normal α2-macroglobulin levels would suggest an absence of vascular inflammation/injury. Similar to troponin, this biomarker is also easily obtainable (serum sample) and measurable (immunoassay).

Natriuretic peptides function to regulate fluid volume, electrolyte balance, and blood pressure. They are released in response to increased blood pressure, increased sodium concentration, and atrial/ventricular stretch. B-type natriuretic peptide (BNP) is a hormone released by the heart during ventricular stress (Lubien *et al*, 2002) . The group recommended BNP as a biomarker to evaluate myocardial pressure and volume overload. BNP is considered a strong "negative predictor" since a low value means circulatory volume parameters are normal. An elevated BNP indicates a change in circulatory volume or blood pressure and may suggest hypervolumic states related to such conditions as heart failure, cardiac hypertrophy, renal disease, liver disease, or certain endocrine disorders. The human serum BNP assay is not appropriate for rodents; therefore, the group suggested that NTP develop a serum BNP assay that is. Currently, in rodents the BNP assay requires RNA extraction from the heart, which limits measurement collection to necropsy.

Ultrasound is a noninvasive technology that can be used on a live animal to detect early and late cardiac disease and can be a relatively high throughput technology (40 - 60 animals a day). Detection of abnormal heart rate and blood pressure fluctuations may predict future heart disease (Badea *et al.*, 2004; Badea *et al.*, 2006; Badea *et al.*, 2005). Molecular probes may be used in conjunction with ultrasound to further understand disease processes. Enhanced imaging techniques, such as a Micro Computed Tomography Scanner (Micro CT), were recommended for use in characterizing disease processes after a cardiac toxicant is identified.

Biomarkers of Lipid/Carbohydrate Metabolism

Metabolic diseases such as diabetes have both genetic and environmental components (Florez *et al*, 2006) . Often the term "metabolic syndrome" is applied to a disease process that may result in insulin resistance or increased risk for cardiovascular disease (Eckel *et al.*, 2005). Obesity is projected to affect over 65% of the adult population in the United States (Muoio and Newgard, 2006), is a common cause of insulin resistance in children, and is associated with an increase in type 2 diabetes and vascular disease (Weiss *et al.*, 2004). High fat diets are linked to tissue dysfunction and disease development (Muoio and Newgard, 2006).   Increased plasma lipid levels may block insulin signal transduction (Muoio and Newgard, 2006) resulting in increases in serum insulin levels.

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The breakout group recommended measurement of serum levels of cholesterol/triglycerides, insulin, and glutathione[2] as useful biomarkers of metabolic alterations possibly leading to disease. The lipoproteins HDL, LDL, and VLDL were not recommended primarily because of differences between rodents and humans. In rodents, cholesterol partitions primarily into the HDL fraction, while in humans, a considerable amount partitions into LDL (Lehmann *et al.*, 1993). HDL is the predominant lipoprotein in mice (LDL is in humans) and increases in HDL in mice do not tend to parallel increases in HDL in humans. Thus, total cholesterol is a more reasonable comparison to make between rodent and human data. The group did not believe measuring individual lipoproteins would provide sufficient added benefit compared to measuring total serum cholesterol. Routine measurement of fatty acid concentrations also was not recommended because they were not considered to be very sensitive or informative unless the animals are in a tightly controlled, fasted state (NTP animals are not typically fasted).

Insulin was recommended because it is considered a better marker of glucose regulation than glucose, particularly in animals that are not fasted. Other alternatives include measuring glucose attached to hemoglobin in red blood cells (hemoglobin A1c or HbA1C) (Saudek *et al.*, 2006) or serum fructosamine (glycated albumin) (Fonseca *et al*, 2000; Monnier, 2006), because they are less sensitive to fluctuations based on feeding status (Rendell *et al*, 1985) .

Body composition analysis using dual-energy x-ray absorptiometry (DXA or DEXA) to evaluate lean mass, fat mass, and bone density or microCT to distinguish visceral and subcutaneous fat was recommended for selective use to identify possible metabolic disease processes (Jamieson *et al.*, 2006). Like the heart breakout group, this group thought general measures of inflammation, such as TNF-alpha and IL-6, could be useful as a routine measurement or in special studies even though changes would not be specific to perturbations in lipid or carbohydrate metabolism.

NTP PERSPECTIVE

NTP convened this workshop to identifybiomarkers for heart, lung, and lipid/carbohydrate disease that could be added routinely to NTP subchronic toxicology studies. Based on discussions at the workshop and follow-up meetings among NTP staff, it is clear for a variety of reasons that very few of the biomarkers recommended are appropriate for routine inclusion in NTP studies. Prior to making any modifications to the toxicology bioassay, the NTP must carefully consider "added value." Added value must consider not only the scientific knowledge gained, but also resources expended (e.g., equipment, additional animals, costs, personnel, etc.) to obtain that knowledge. While all of the biomarkers discussed have scientific merit, any included in an NTP study paradigm must possess three critical scientific attributes: (1) appropriate for the species being tested (i.e., rats and/or mice), (2) sensitive and specific for detecting injury or

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[2] Although glutathione was recommended by the lipid/carbohydrate breakout group, this endpoint was discounted during final plenary discussions as being too non-specific, and problematic to measure correctly.

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altered function, and (3) relevant for identifying potential human health effects. If a biomarker possesses these attributes, then NTP must consider other more practical factors such as invasiveness of sampling procedure, availability of a reliable, rapidly performed assay or technique, and cost-worthiness.

Biomarkers of Lung Function and Injury

Given the challenges of studying the respiratory system, it is not surprising that the lung breakout group did not strongly recommend any biomarker for routine inclusion in NTP studies. The most promising techniques identified were BAL and enhanced histopathology. The NTP does not consider either of these necessarily appropriate for studies that do not use inhalation as the route of exposure unless the lung is a known target organ. The use of BAL in inhalation studies would increase the number of animals used per study and require some degree of methods development and staff training. The most likely scenario is that NTP will continue to use BAL and enhanced histopathology for special studies when the lung is a suspected target, and, the specific research question will guide and ultimately determine which specific markers NTP measures using these techniques.

Biomarkers of Heart Function and Injury

The NTP is currently developing and validating a serum troponin assay for routine use as a screen for cardiac damage. While the breakout group did not recommend a specific type of troponin (i.e., TnT or TnI), the NTP is validating an assay based on TnT and plans initially to run all these assays at the NIEHS. Currently only one vendor has appropriate reagents. The use of a single assay in one laboratory should result in consistent assay performance. The current assay for TnT appears to perform well in the rat, although the NTP will evaluate the mouse as well. To validate this assay, serum samples will be collected after exposures to known cardiotoxicants and compared to heart histopathology to determine if the assay can be used to detect early signs of cardiotoxicity. Additionally, the NTP will compare troponin response and cardiac histology for about 20 chemicals to evaluate its predictive and diagnostic utility.

The NTP is also interested in supporting research to develop a serum assay for B-type natriuretic peptide because the current assay requires RNA extraction from the heart, which limits collection to necropsy. Enhanced pathologic analysis of cardiac tissue and electron microscopy may also be of particular help for identifying injury as has been shown with AZT (Bishop *et al*, 2004) for identifying development of mitochondrial damage (Dunnick *et al*, 2004) . This enhanced pathology is particularly important in order to identify subcellular structural abnormalities and would be used for special studies.

Biomarkers of Lipid/Carbohydrate Metabolism

Since the workshop, the NTP has added total serum cholesterol and triglyceride concentrations to its clinical chemistry panel. While the breakout group recommended

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that NTP routinely measure insulin as a marker of abnormal carbohydrate regulation, the NTP believes a more practical option is fructosamine, an indicator of long term glycemic control. The NTP considers fructosamine to be a more practical option for measuring glycemic control than HbA1C because the rate of HbA1C formation is variable between species due to differences in erythrocyte glucose permeability (Rendell *et al.*, 1985). In comparison to the insulin immunoassay, the assay for fructosamine is considerably less expensive and more amenable to routine use. The NTP is currently validating a fructosamine assay.

Inflammation biomarkers

The importance of assessing inflammation in toxicology studies was a reoccurring theme in all breakout groups because of its role in multiple disease states. Some biomarkers may be useful for detecting a variety of disease processes including markers for inflammation such as chemokines biomarkers (Charo and Ransohoff, 2006), which direct circulating leukocytes to sites of inflammation, or TNF-alpha or interleukins. NTP is exploring the feasibility of adding a panel or profile of selected markers of inflammation.

Imaging Analysis

While non-invasive imaging technologies are appropriate for each type of health effect considered at the workshop (i.e., x-ray, MRI, or PET scanning for lung disease, heart ultrasound, body composition analysis with dual-energy X-ray absorptiometry, etc.), the NTP will not be incorporating these technologies on a routine basis at the present time. Regular use would require the purchase of new equipment and hiring trained personnel. In addition, imaging technologies may also require centralized facilities because of the large fixed costs in buying and maintaining equipment. All of these factors combined make routine use cost-prohibitive. However, NTP and NIEHS are using these technologies in small pilot studies to identify lung and heart disease in rodents.

Gene Expression Data

All three breakout groups considered gene expression analysis. Gene expression analysis has been used on human tissues in an attempt to characterize and predict disease processes (Blaxall *et al*, 2003; Wesselkamper *et al.*, 2005). And, thus, gene transcript changes from rodent toxicology studies could be evaluated with a vision to extrapolate the findings as they might relate to human disease. Advantages of gene expression data are that analysis allows for measuring indicators that are part of multiple disease processes. However, to interpret the data, it is necessary to correlate genotypic and phenotypic changes and relate these changes both within a species and between species (e.g., rodents and humans). The bioinformatics required to interpret the data properly are not as advanced as the technology, which prohibits the use of gene expression data on a large scale. In addition, the bioinformatics methods will require more standardization before the information can be used in regulatory decisions (Goodsaid and Frueh, 2006). In summary, while gene expression analysis was recognized as a valuable research tool, none of the breakout groups felt it is appropriate for routine inclusion in NTP studies at

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the present time. The groups suggested, however, that NTP consider selectively archiving tissue such as the spleen, liver, heart, lung, and possibly brain for future gene expression analysis as part of its routine toxicology studies.

SUMMARY AND CONCLUSIONS

In summary, NTP felt the workshop provided valuable insights into biomarkers that would address information about lung and cardiac function and injury as well as lipid/carbohydrate metabolism and greatly appreciated the active participation by all workshop attendees. After careful consideration of the workshop's recommendations, the NTP will immediately begin including serum cholesterol and triglycerides as routine measures in its subchronic studies. Several other biomarkers (TnT, fructosamine, and possible BNP) will be added routinely when the assays are appropriately standardized and validated. Initially, NTP will limit routine collection of samples for biomarker analysis to the rat because the rat can provide more sample volume whereas routine collection in mice may not be feasible unless additional animals are used. Other recommended biomarkers (e.g., imaging, BAL, gene expression, etc.) will included as adjunct evaluations on a more limited basis.

Use of biomarkers for heart, lung, or altered lipid/carbohydrate metabolism will likely require internal validation studies with known chemicals to determine the utility of a biomarker for routine hazard identification studies. In addition, as an integral component of the current effort NTP plans to conduct an assessment in 5 to 10 years on whether the addition of the biomarker endpoints enhances our ability to detect and understand environmentally-induced diseases.

Acknowledgement:

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